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Characterization of a 22-residue peptide derived from a designed ion channel

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Abstract

We have designed a four-helix protein that is expected to tetramerize in the membrane to form an ion channel with a structurally well-defined pore. This should serve as a model system to study the structural requirements of voltage-sensitive, ion-selective transmembrane channels. We have synthesized the peptide corresponding to the channel-lining helix. Circular dichroism (CD) spectroscopy shows that this peptide is helical in the membrane. Fluorescence resonance energy transfer (FRET) shows that this peptide, at low concentrations, forms aggregates in 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) liposomes and facilitates ion transport across liposomal membranes. Our data indicate that a component of the designed four-helix protein, i.e., the channel-lining helix, behaves as per design. © 1997 Elsevier Science B.V.

Keywords: Protein design; Peptide synthesis; Ion transport; Liposome; Circular dichroism; Fluorescence resonance energy transfer

1. Introduction

The three-dimensional structure of a protein is dictated by the sequence of its constituent amino acids, but it has not thus far been possible to develop reliable algorithms leading from a primary sequence to its three-dimensional structure. One approach to this problem has been to design proteins based on the propensity of certain amino acid residues to adopt preferred conformations. Four helix bundles such as FELIX have been so designed [1]. Other attempts on the same lines have been less successful [2]. Indeed, a common failing of synthetic proteins appears to be that while they may adopt secondary structures as predicted, their tertiary structures are very poorly defined [3]. Introducing constraints such as metal binding sites has led to better defined structures [4]. More recently, it has been possible to design proteins without such constraints that do adopt the desired structures [5,6]. Membrane proteins are constrained to adopt a limited range of secondary structures. Once a polypeptide chain enters the membrane phase, polar groups on its backbone are no longer able to hydrogen bond with solvent and consequently are forced into hydrogen bond interactions with other backbone polar groups. Shielding would be maximized by either helical or β-structures. Structural data on the few

Abbreviations: ALH, resin with acid labile handle; AcPCH3, acetylated PCH3; CD, circular dichroism; CTC, chlortetracycline; Dans, dimethylaminonaphthylsulfonamide; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; FRET, fluorescence resonance energy transfer; PCH3, peptide corresponding to helix no. 3; PCH3-Dans, dansyl derivative of PCH3; UV, ultraviolet; X537A, laslocid

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membrane proteins studied to date are consistent with such an analysis [7–9]. The determinants of tertiary structure in such cases are, however, unclear.

The *Shaker* K^+-channels represent a family of voltage-gated potassium-selective ion channels [10–12]. The functional channel has four subunits, each having six putative transmembrane segments [12] with contributions to the pore liner coming from all four subunits [13,14]. Formation of a stable pore with a well-defined conductance in such a protein requires that tertiary interactions stabilize the pore. While the structural basis of the selectivity filter in these proteins is not well understood, it is likely that it would require the side chains of the constituent residues to adopt a very precise three-dimensional arrangement. Thus, designing a protein that will tetramerize in the membrane and form channels with a well-defined conductance requires designing a protein with a well-defined tertiary structure.

We have designed a protein, SYNCHAN, that should tetramerize in membranes to form a non-selective, ungated pore (Seth et al., in preparation). The channel liner for this protein is an amphipathic helix, the polar face of which is based loosely on the alamethicin–zervamicin family of fungal peptide antibiotics [15]. The other faces have been designed based on bacteriorhodopsin helices known to associate strongly with each other [16]. In this communication, we report on the ability of a peptide corresponding to this de novo designed channel liner to mediate ion transport through liposomal membranes and characterize the structural basis for channel formation by this peptide.

2. Materials and methods

2.1. Materials

A polyamide resin with an acid labile handle (ALH) was purchased from Ultrosyn (Ultrosyn ResinA), LKB Biochrom Ltd. (Cambridge, UK). All Fmoc-Amino acids, (OPfp esters except for Ser and Thr, DHBT esters for Ser and Thr), hydroxybenztriazole (HOBT), dimethylaminopyridine (DMAP) were from Novasyn-Novabiochem (Nottingham, UK). Amino acids, 1-dimethylamino-naphthalene-5-sulfonic acid (dansic acid), chlortetracycline (CTC), laslocid (X537A) and dimyristoylphosphatidylcholine (DMPC) were from Sigma. Tertiary amyl alcohol was from Ultrosyn, trifluoroacetic acid (TFA) from Sigma and dicyclohexylcarbodiimide (DCC), ethanedithiol (EDT), anisole were from Merck, India. Dimethylformamide (DMF), dichloromethane (DCM), acetonitrile, methanol, acetone were from Ranbaxy, India, and diethylether from Qualigens, India. All solvents were distilled prior to use. Soya-lecithin was obtained from a local source, and recrystalized from acetone.

2.2. Peptide synthesis and acetylation

The peptide was synthesized on a flow synthesizer (LKB Biochem-Biolynx 4175) on a scale of 1 g of Ultrosyn ResinA (0.1 mmol) using Fmoc chemistry [17]. The first residue was attached using Fmoc-Trp-OPfp ester. A part of the peptide was acetylated at its N-terminus, using acetic anhydride [17] before cleavage from the resin.

2.3. Dansylation of the peptide

Another part of the peptide was dansylated at its N-terminus, using dansyl chloride. Recrystalized dansyl-chloride was prepared following the procedure described [18]. In brief, 1 g of dansic acid was taken in 3 ml of phosphorous-oxychloride ($POCl_3$) and 1.6 g of phosphorous pentachloride (PCl_5) added slowly until the mixture turned homogeneous. The resulting mixture was poured into $\sim$80 g of ice; extracted with benzene, washed with dilute alkali, dried over calcium chloride and the benzene evaporated off. The resulting reddish-orange oil solidified on storing at -20°C. Finally it was recrystalized from light petroleum ether. The peptide was dansylated at its free N-terminus on the resin prior to cleavage and deprotection as follows. One hundred milligrams of resin was taken in 3 ml of dry methanol. Ten milligrams of freshly prepared dansylchloride and 5 ml of triethylamine (TEA) were added and the mixture incubated at room temperature overnight. Excess dansylchloride was washed off with methanol followed by diethylether. Cleavage and deprotection were carried out by standard methods.

The peptide and its derivatives were checked for purity by analytical HPLC (Shimadzu SPD6a) on a

C_{18} reversed phase column. Two dimensional NMR studies (400 mHz) of the undansylated peptides confirmed their composition and sequence. The peptide/derivatives synthesized were:

PCH3, YC(Acm)FALQFAPSLITFTVFLLTLW;
PCH3-Dans, PCH3 dansylated at the N-terminus;
AcPCH3, PCH3 acetylated at the N-terminus.

2.4. Fluorescence spectroscopy

Fluorescence spectra were recorded on a SPEX Fluorolog 2 with 1.8 nm excitation bandpass and 1.25 nm emission bandpass with a step size of 0.5 nm per point and an integration time of 1.0 s. Four scans were averaged for each spectrum. Energy transfer efficiencies were estimated from emission spectra recorded from equal amounts of dansylated and undansylated peptide excited at 285 nm. The decrease of emission intensity at the tryptophan peak is related to the efficiency of energy transfer.

$$E = \text{Transfer efficiency} = (1 - F_{DA}/F_D) \quad (1A)$$

where F_D and F_{DA} are the emission intensities of tryptophan in absence and presence of the acceptor, respectively. Assuming free rotation of both the donor and the acceptor, the distance between the two is given by

$$R = R_0(E^{-1} - 1)^{1/6} \quad (1B)$$

where R_0 is the distance at which the efficiency is 50% [19]. R_0 is reported to be 23.0 Å for the tryptophan–dansyl pair [20].

2.5. CD spectroscopy

CD spectra were recorded on a Jasco 720A spectropolarimeter after the instrument was calibrated with (+)-10-camphorsulfonic acid. A 1-cm pathlength cell was used and spectra scanned at 50 nm/min in steps of 0.025 nm. Sixty-four scans were averaged for each spectrum. Peptide concentrations were in the range of 1–3 μM. The mean residue elipticity was estimated as:

$$[\theta] = \theta/(10 \times \text{Concentration in molarity} \times \text{pathlength in cm} \times \text{no. of residues}) \quad (2)$$

2.6. Preparation of small unilamellar vesicles

Small unilamellar vesicles (SUV) were prepared following the procedure described in [21] except that soya-lecithin or DMPC was used. Briefly, dry lipid was dissolved in a chloroform/methanol mixture (3:1, v/v). The solvents were evaporated under a stream of nitrogen until a thin film was deposited on the walls of the test tube. The thin film thus obtained was then resuspended in 5 mM HEPES buffer (pH 7.0) containing 100 mM NaCl. The resulting lipid dispersion was then sonicated using a microtip probe (Virsonic 475) until optical clarity was achieved. The clear suspension was centrifuged at $100\,000 \times g$ for 1 h at 4°C and the upper 2/3 of the supernatant used for subsequent measurements. The final concentration of lipid was 15 mg/ml.

2.7. Ion-transport measurements

Ion transport was assayed fluorimetrically on a SPEX Fluorolog 2 as described in [22]. Briefly, 100 μl of the liposome suspension was taken in a 3 ml fluorescence cuvette along with 10 μl of 2.3 mM CTC solution in de-ionized water. After incubation for 2–3 min, the mixture was diluted with 1.8 ml of buffer and stirred continuously while monitoring at $\lambda_{ex} = 400$ nm and $\lambda_{em} = 530$ nm. Fifty microliters of 40 mM $CaCl_2$ solution was added after collecting a baseline. Test samples were delivered through an injection port as a methanolic or aqueous solution after a steady state had been achieved. An influx of calcium into the liposomes mediated by the injected sample can be detected as an increase in the intensity of CTC fluorescence.

3. Results and discussion

3.1. Design of SYNCHAN

We have designed a four-helix protein that should tetramerize in the membrane to form ion-conducting pores. The protein, SYNCHAN, should thus serve as a model for *Shaker*-type potassium channels which are also tetrameric. Fig. 1A shows a schematic cross-section of the functional channel. The design requires that helix 4 associates with helix 2, helix 3,

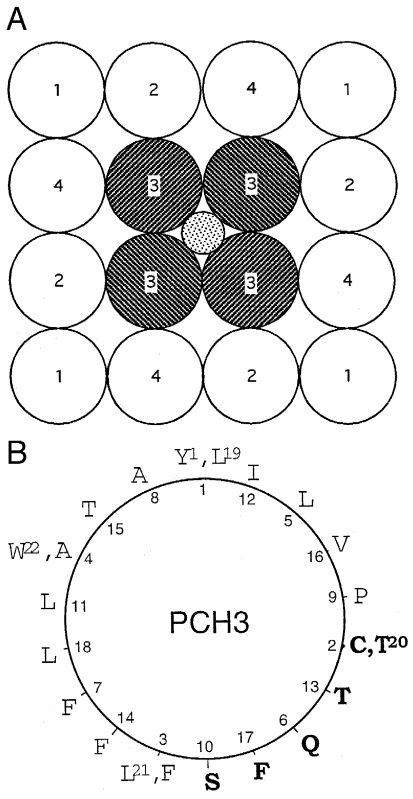

Fig. 1. A: the synthetic channel is designed to be a tetramer of a protein with 4 transmembrane helices, one of which (no. 3 here) lines the channel. The cross section of the assembled tetramer is shown, with the channel liner hatched. The central pore is also indicated in this panel. B: helical-wheel representation of the peptide PCH3, viewed down the helix axis. The arc from Cys^2 to Ser^{10} with residues in boldface is designed to line the aqueous pore.

and lipid; while helix 3 should associate with helix 2 and helix 4 and also line the channel. Faces of helices that interact extensively in the bacteriorhodopsin structure [8] have been used in the design. The interacting faces from helices A and B in the bacteriorhodopsin structure have been transposed onto the helix 1–helix 4 pair in SYNCHAN. Other interacting faces of SYNCHAN were designed in an analogous manner using other interacting pairs of helices of bacteriorhodopsin. Faces of helices that are exposed to lipid have been made up of predominantly hydrophobic residues, while the channel-lining face of helix 3 has been loosely modeled on the alamethicin–zervamicin family. It should be emphasized that while individual faces of helices have been based on the structure of bacteriorhodopsin, an optimal alignment of SYNCHAN and bacteriorhodopsin sequences yields only 25 matches out of 130 SYNCHAN residues. It is thus a protein with very limited homology to any known sequence.

The channel-lining helix, helix 3, interacts with helices 2 and 4 of the same subunit, helix 3's of the neighboring subunits and the aqueous pore. It has been designed as an amphipathic helix with five out of the six residues lining the pore being polar (Fig. 1B). The peptide corresponding to helix 3 (PCH3) was synthesized by solid phase peptide synthesis. In analogy to the alamethicin–zervamicin family, the peptide is expected to insert into the membrane and form helical aggregates therein which enclose an aqueous pore that can mediate ion transport [23].

3.2. CD and fluorescence studies of structure

The CD spectrum of AcPCH3 in the presence of DMPC liposomes (Fig. 2) shows two negative bands characteristic of α-helical conformation. Since the CD spectra of small peptides containing aromatic residues reflect the algebraic sum of contributions from the peptide bonds and from the aromatic residues, estimates of fractional helicity from such spectra should be viewed with caution [24,25]. For the same reason, we have not used any curve deconvolution programs to estimate fractional helicity in

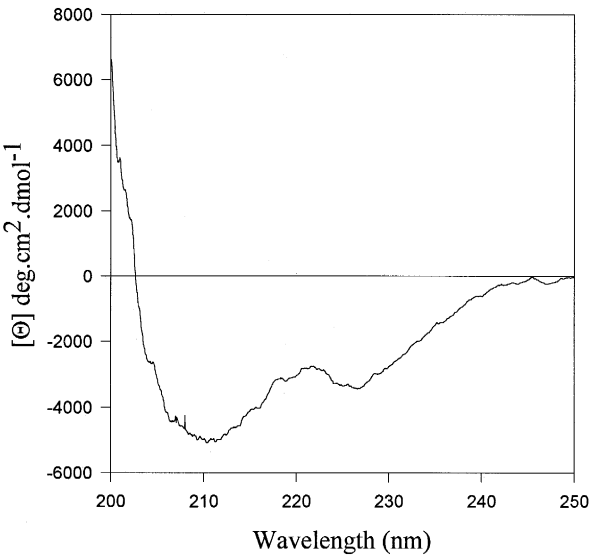

Fig. 2. Far-UV CD spectra of AcPCH3, 1.25 μM, in liposomes at 25°C. The lipid concentration is 0.75 mg/ml.

this peptide. We have investigated the structure of the peptide in methanol–water systems and in liposomes and shown that it is α-helical in methanol and liposomes, but adopts β-structures in aqueous buffer [26]. The observed CD spectrum of the peptide in liposomal suspensions appears α-helical, implying that the peptide has inserted into the membrane [26].

PCH3 is intrinsically fluorescent with a tyrosine at its N-terminus and a tryptophan at its C-terminus. We have also prepared a dansylated derivative with the dansyl group attached to its N-terminus (PCH3-Dans). The emission spectrum of the dansyl group is very sensitive to its environment, the emission maximum shifting from around 560 nm in aqueous solution to shorter wavelengths in apolar media [22]. The emission spectrum of tryptophan is also sensitive to solvent polarity, but the wavelength range is much more restricted. Fig. 3 presents emission spectra of PCH3 and PCH3-Dans in HEPES buffer and in liposomes. On direct excitation of the dansyl group, the emission maximum of PCH3-Dans shifts from 500 nm in HEPES buffer to 490 nm in liposomes (Fig. 3A). This would indicate that the peptide forms aggregates in water that effectively shield the chromophore from solvent. Insertion into the bilayer results in the chromophore sensing an even less polar environment within the membrane phase. Aqueous phase aggregates of the peptide would be expected to sequester the apolar faces, thus shielding them from the solvent while membrane phase aggregates, if formed, would expose the apolar faces to lipid. The position of the emission peak of the Dans was essentially invariant over the concentration range from 0.7 to 5.25 μM, indicating that it is aggregated throughout this range.

Excitation of tryptophan at 285 nm leads to either emission from tryptophan or resonance transfer of energy to the dansyl group and subsequent emission from that chromophore. The efficiency of this phenomenon falls off with the sixth power of distance and is 50% efficient at a distance of 23.0 Å for the Trp–Dans pair [20]. In aqueous solution (Fig. 3B), the presence of the dansyl group decreases the emission intensity of tryptophan by 34% for a peptide concentration of 0.7 μM with a concomitant increase in emission at the dansyl wavelengths. The distance corresponding to this efficiency of energy transfer is 25.7 Å which is less than the 33 Å predicted for a 22-residue α-helical peptide. This is unlikely to arise

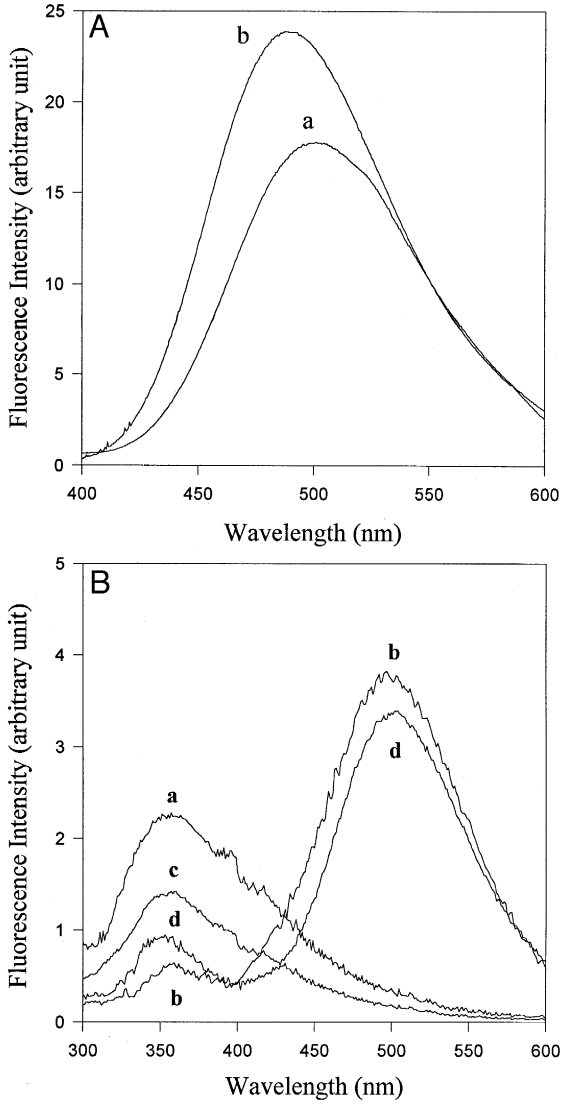

Fig. 3. A. Fluorescence emission spectra of PCH3-Dans, 1.25 μM in (a) HEPES buffer (pH 7.0) at 25°C, and (b) liposomes at 35°C (lipid concentration 0.75 mg/ml). Excitation wavelength is 350 nm. B. Fluorescence emission spectra of AcPCH3, 0.7 μM (a) and PCH3-Dans, 0.7 μM (b) in liposomes at 35°C (lipid concentration 0.75 mg/ml); AcPCH3, 0.7 μM (c) and PCH3-Dans, 0.7 μM (d) in HEPES buffer (pH 7.0) at 25°C.

from a population of monomeric peptides with an average end-to-end distance of 25.7 Å as the dansyl emission clearly indicates that the peptide is aggregated. Conversely, interchromophore distances are likely to be much shorter than 20 Å in helical, antiparallel aggregates. Centrifugation at $100\,000 \times g$ for 1 h did not affect the spectra recorded, indicating that the aggregates formed are small.

The energy transfer efficiency in liposomes is much higher (75%) indicative of an average inter-chromophore spacing of 19 Å which is significantly less than the bilayer thickness. Aqueous phase aggregation of the peptide at this concentration resulted in only 34% energy transfer indicating either that some of the peptide is monomeric under these conditions or that the structures of the aggregates in water and in membranes are very different. The peptide is helical in the membrane (Fig. 2), making intramolecular transfer unlikely. We, therefore, interpret this data as indicative of antiparallel aggregation in the membrane. For peptide at a concentration of 0.7 μM dispersed in 0.75 mg/ml lipid with an average head group diameter of 10 Å [27], the average spacing between peptide molecules would be expected to be ~105 Å, if peptide molecules were randomly distributed in lipid bilayer. No energy transfer is expected at distances greater than 45 Å for the Trp–Dans pair.

3.3. Ion-transport activity

The ability to facilitate the transport of calcium across liposomal membranes is assayed using the calcium indicator dye chlortetracycline (CTC) entrapped within liposomes with calcium present in the extravesicular buffer. An increase of fluorescence intensity on adding peptide or a known ionophore is indicative of an influx of calcium. The integrity of the vesicles is checked with the calcium ionophore X537A (Fig. 4A). The rise in fluorescence intensity of CTC observed on adding the ionophore requires that CTC and Ca^{2+} are in separate compartments. Fig. 4B shows that PCH3 is unable to facilitate ion transport at a concentration of 3.75 μM when added from a methanolic solution. Addition of the same concentration of AcPCH3 from a methanolic solution results in a rise in CTC fluorescence indicative of ion transport. On the other hand, addition of 5.25 μM AcPCH3 from an aqueous solution is ineffective. However, PCH3-Dans facilitates ion transport at much lower concentrations than AcPCH3 (data not shown). The efficacy of methanolic solutions of PCH3 derivatives parallels their hydrophobicity with PCH3-Dans being more hydrophobic than AcPCH3 which, in turn, is more hydrophobic than PCH3 free base.

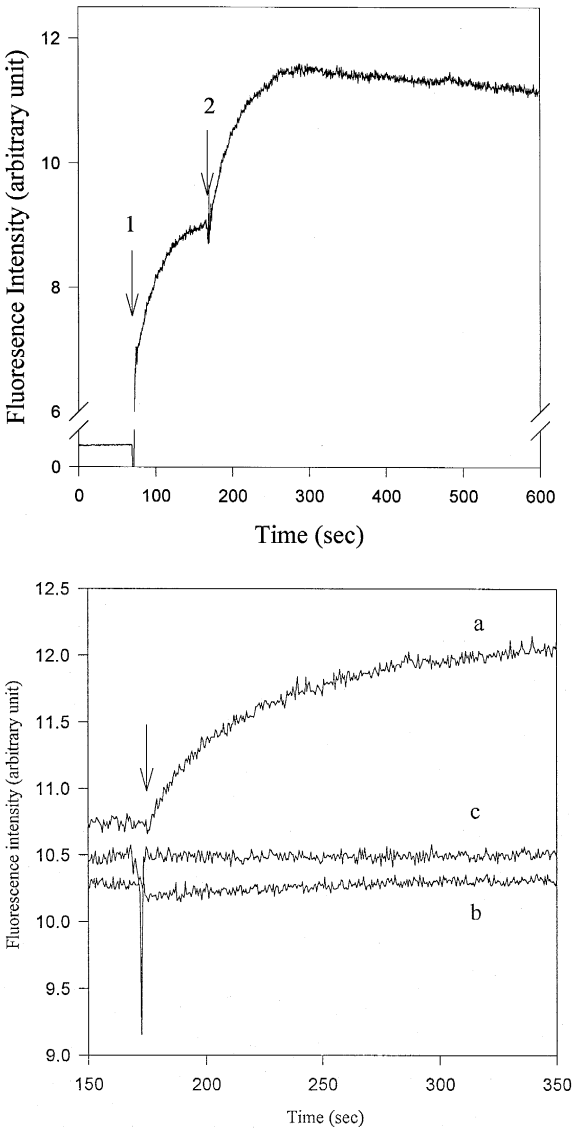

Fig. 4. A: ion transport across liposomal membranes: $CaCl_2$ is added at the point indicated by arrow 1; arrow 2 indicates the addition of the ionophore, X537A. B: ion-transport activity of: (a) methanolic AcPCH3 (3.5 μM); (b) aqueous AcPCH3 (5.25 μM); and (c) methanolic PCH3 (3.75 μM). The peptide is added at the point indicated by the arrow. The lipid concentration is 0.75 mg/ml.

Addition of AcPCH3 to liposomal suspensions from methanolic stock results in largely helical peptide inserted into the membrane, whereas addition from aqueous stock leads to disordered peptide [26]. An amount of 1.25 μM AcPCH3 added from methanolic stock mediates ion-transport activity, whereas even 5 μM peptide from aqueous stock is

inactive (Fig. 4B). The lack of activity of the aqueous stock argues against detergent action leading to permeabilization. Conversely, the correlation between the presence of helical peptide in the membrane and ion-transport activity is consistent with the designed role of the peptide forming helical aggregates enclosing aqueous pore.

Channel-forming activity in alamethicin fragments increases with chain length and overall hydrophobicity, the minimum concentration at which activity is detected scaling with the critical 'micellar' concentration (cmc) for dansylated derivatives [28]. These data were interpreted to indicate that alamethicin inserts into membranes as preformed aggregates which rearrange in the membrane to form channels [23,28]. In the case of PCH3, the free base is inactive, the acetyl derivative is active at concentrations down to 1.05 μM while the dansyl derivative is active down to 0.3 μM (data not shown). Hence, it seems reasonable to conclude that our data with PCH3 and its derivatives are consistent with such a model for channel formation.

The ion-transport activity of AcPCH3 increases with concentration. Fig. 5A shows traces for the assay run at a range of concentrations from 0.7 to 5.25 μM. The initial slopes of these curves is a good indicator of ionophore activity [29] and this parameter is plotted against concentration in Fig. 5B. Ionophore activity can be seen down to at least 1.05 μM. The initial slope of fluorescence increase caused by the authentic ionophore, laslocid, at a concentration of 20 μM is comparable to that seen with 4.9 μM AcPCH3.

We have not estimated the sizes of the liposomes used in our assay. However, SUVs prepared by sonication have been reported to range in size from 100 to 500 Å in diameter [30]. An amount of 1.25 μM peptide is clearly effective in mediating ion transport in liposomes at 0.75 mg/ml: a ratio of 1 peptide per liposome, assuming a mean liposome size of 300 Å. Increasing the peptide concentration increases the probability of channel formation in a given liposome which is reflected in enhanced initial rates of fluorescence increase and greater limiting amplitude of the response. Given that at least 4 monomers would be required in a liposome to form a channel, these data are consistent with a channel mechanism for permeabilization.

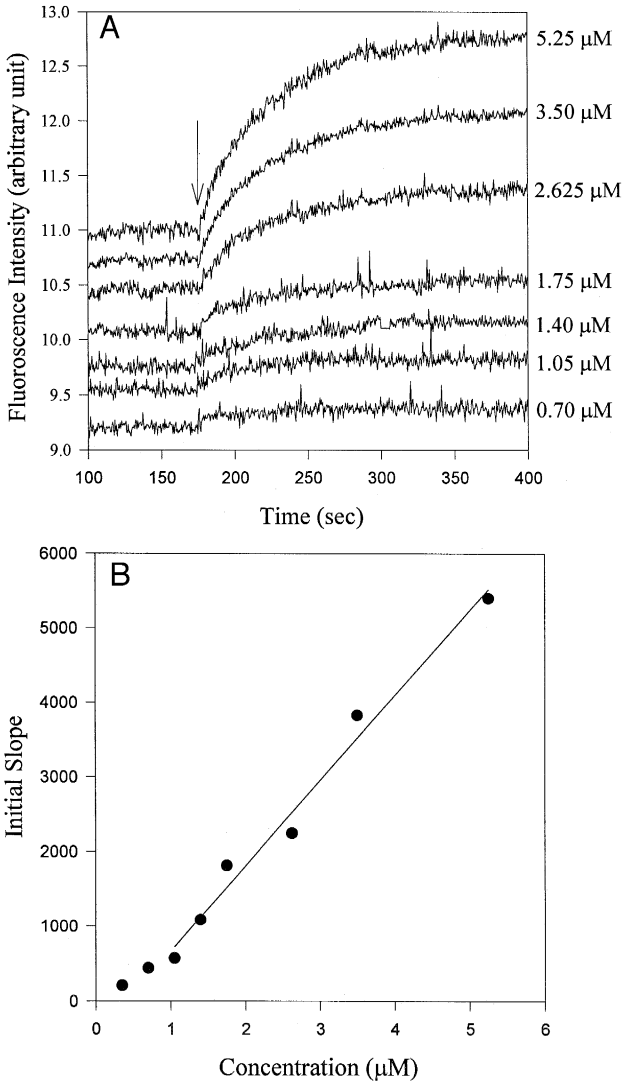

Fig. 5. A: ion-transport activity of AcPCH3 across liposomal membrane. The peptide is added at the point indicated by the arrow. Concentrations used are indicated against the traces. The lipid concentration is 0.75 mg/ml. B: the initial slope of the rise in fluorescence following peptide addition is plotted against the final concentration of the peptide added.

In summary, the peptide PCH3 which corresponds to helix 3 of the design for SYNCHAN forms helical aggregates in liposomal membranes that mediate transmembrane ion transport. Ease of aqueous phase aggregation parallels the ability to form channels, consistent with the requirement for insertion of preformed aggregates into the membrane to form channels. These properties of the peptide match those predicted by the design, encouraging further development of the SYNCHAN protein.

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